DEVELOPMENT OF A RATIONALLY-DESIGNED, LOW ABUSE POTENTIAL, BIOGENIC AMINE RELEASER THAT SUPPRESSES COCAINE SELF-ADMINISTRATION


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Non-standard abbreviations: PAL-287 (1-naphyl-2-aminopropane), (±)-MDMA ((±)-3,4-methylenedioxymethamphetamine), PAL-313 (para-methylamphetamine), dopamine (DA), NE (norepinephrine), 5-HT (serotonin), DAT (DA transporter), SERT (5-HT transporter), NET (NE transporter), FR (fixed ratio).
Abstract

Convergent lines of evidence support a dual deficit model of stimulant withdrawal, where reductions in synaptic dopamine (DA) and serotonin (5-HT) contribute to dysphoria, drug craving, and relapse. Thus, we predicted that a non-amphetamine compound with substrate activity at DA and 5-HT transporters (i.e., a dual DA/5-HT releaser) would be an effective medication for treating stimulant addictions. Ideally, this type of medication would alleviate withdrawal symptoms, suppress cocaine self-administration, and lack side effects commonly associated with CNS stimulants. In the present work, over 350 compounds were screened in vitro for activity as substrate-type releasing agents at DA, 5-HT and norepinephrine (NE) transporters. These efforts identified PAL-287 (1-napthyl-2-aminopropane) as a non-amphetamine compound with potent substrate activity at biogenic amine transporters. In vivo microdialysis in rats demonstrated that PAL-287 (1-3 mg/kg, i.v.) increased extracellular DA and 5-HT in frontal cortex, but effects on 5-HT were somewhat greater. PAL-287 induced substantially less locomotor stimulation than (+)-amphetamine, a drug which increases only extracellular DA. Administration of high-dose (+)-methamphetamine or (±)-MDMA to rats produced long-lasting depletion of cortical 5-HT, whereas PAL-287 (18 mg/kg, i.p. X 3) did not. PAL-287 displayed little or no reinforcing properties in rhesus monkeys trained to self-administer cocaine, yet PAL-287 produced a dose-dependent decrease in responding for cocaine when infused at a dose of 1.0 mg/kg/hr. Collectively, the findings reported here demonstrate that non-amphetamine monoamine releasing agents like PAL-287 might be promising candidate medications for the treatment of stimulant dependence.
Introduction

The abuse of illicit stimulants like cocaine and methamphetamine remains a major public health problem in the United States (Banken, 2004). Importantly, the abuse of CNS stimulants negatively impacts public health, through the spread of HIV-1, hepatitis B and C, and drug resistant tuberculosis (Kresina et al., 2004). Despite the extent of the stimulant abuse crisis, development of medications to treat stimulant dependence has not been successful (Shearer and Gowing, 2004).

The lack of effective medications for stimulant dependence renders discussions of an “ideal” pharmacotherapeutic for stimulant addiction somewhat hypothetical. Nevertheless, a reasonable conceptualization distinguishes between medications that reduce drug-seeking behavior, and medications that reduce relapse (van den Brink and van Ree, 2003). The former can be readily tested in animal models that measure drug self-administration, whereas the latter can be tested in models which are somewhat less validated (Katz and Higgins, 2003). It seems possible that medications that reduce drug-seeking behavior might also prevent relapse due to shared neurochemical properties between the medication and the abuse d stimulant. Agonist substitution therapy illustrates this approach. Often described as neurochemical normalization therapy (Rothman et al., 2002a), agonist substitution therapy has generated effective treatments for nicotine dependence (Henningfield, 1995) and opioid dependence (Kreek, 1996).

We, and others, have advocated the use of monoamine releasers like amphetamine as ‘agonist’ therapies for stimulant dependence (Rothman et al., 2002a; Grabowski et al., 2004). Preclinical studies strongly support this approach (Wojnicki et al., 1999). Negus and Mello (Negus and Mello, 2003a), for example, demonstrated that (+)-amphetamine infusions decrease cocaine self-administration behavior in monkeys,
with minimal effects on food-maintained behavior. Perhaps more importantly, Grabowski et al. (Grabowski et al., 2001) and Shearer et al. (Shearer et al., 2003) showed that (+)-amphetamine is an effective treatment adjunct for reducing illicit cocaine use in cocaine-dependent human patients.

A major impediment to the use of amphetamine-type drugs as treatments is their high abuse potential. A wealth of information has established that DA release from mesolimbic nerve terminals mediates the motor stimulant and addictive properties of drugs like amphetamine (Wise, 1996). We suggested the possibility of designing novel monoamine releasers with reduced abuse potential; this could be accomplished by altering the relative potency of these drugs at DA transporters versus 5-HT transporters (Rothman and Baumann, 2000). The rationale for this idea is based on the knowledge that 5-HT releasing agents like fenfluramine lack stimulant properties and are not self-administered (Woods and Tessel, 1974). Additionally, fenfluramine reduces locomotor and rewarding actions of DA releasers (Bendotti et al., 1980; Baumann et al., 2000; Rothman and Baumann, 2000). Studies using co-administered phentermine and fenfluramine demonstrate that agents which increase both extracellular DA and 5-HT in rat CNS produce minimal motor activity (Baumann et al., 2000) and are not rewarding (Rea et al., 1998) or reinforcing (Glatz et al., 2002). Furthermore, the phentermine/fenfluramine mixture suppresses cocaine self-administration in rodents and non-human primates (Glowa et al., 1997; Glatz et al., 2002). Consistent with other findings (Walsh and Cunningham, 1997), the aforementioned data support the hypothesis that elevations in synaptic 5-HT can counteract stimulant and reinforcing effects mediated by elevations in synaptic DA (Daw et al., 2002; Burmeister et al., 2004).
In the present study, we sought to identify a non-amphetamine agent that evokes transporter-mediated release of DA and 5-HT with similar potency in nervous tissue. The resulting compound, PAL-287, was tested using a variety of in vivo bioassay methods including intracerebral microdialysis in rat cortex, motor stimulant activity in rats, and drug self-administration behavior in rhesus monkeys. Our findings reveal that PAL-287 is a monoamine releaser capable of suppressing cocaine self-administration, with minimal neurotoxic and reinforcing actions.
Methods

Animals.

For in vivo microdialysis experiments, male Sprague-Dawley rats, purchased from Charles River Laboratories (Wilmington, MA), weighing 280-320 g were single-housed (lights on: 0700-1900 h) with food and water freely available. For in vitro neurotransmitter release assays and neurotoxicity studies, rats were group-housed. Rats were maintained in facilities accredited by the American Association of the Accreditation of Laboratory Animal Care, and the procedures described herein were carried out in accordance with the Animal Care and Use Committee of the National Institute on Drug Abuse (NIDA) Intramural Research Program (IRP).

All rhesus monkeys (Macaca mulatta) studied at the University of Mississippi Medical Center and at the Alcohol and Drug Abuse Research Center, McLean Hospital were maintained in accordance with guidelines provided by the NIH Committee on Laboratory Animal Resources. Each protocol was reviewed and approved by the Institutional Animal Care and Use Committee.

Synthesis of 2-(2-propylamine)-naphthalene hydrochloride (PAL-287)

2-Naphthaldehyde (11.0 g, 0.0704 mol) was added to a stirred mixture of ammonium acetate (5.42 g, 0.0704 mol) in nitroethane (101 mL) at 120°C under nitrogen. After refluxing overnight, the reaction mixture was cooled to room temperature and excess nitroethane was removed under reduced pressure. The resulting orange/brown solid was dissolved in a solution of 10% methylene chloride in diethyl ether (30 mL) and washed with brine (2 x 75 mL) and 1.0 M HCl (3 x 50 mL).
organic layer was dried over anhydrous sodium sulfate, filtered through celite, and volatiles were removed under reduced pressure affording 12.2 g (81%) of crude product, which was pure enough for the next reaction. \(^1\)H NMR (CDCl\(_3\)) \(\delta\) (ppm): 8.26 (s; 1H, CH\(_{2}\)CH\(_3\)NO\(_2\)), 7.91 (m; 4H, ArH), 7.56 (m, 3H, ArH), 2.55 (s, 3H, CHCCH\(_3\)NO\(_2\)).

To a stirred solution of 2-(2-nitro-propenyl)-naphthalene (12.2g, 0.0568 mol) and methylene chloride (65 mL) under nitrogen at room temperature in a 500 mL round bottom flask equipped with a reflux condenser and addition funnel, 1.0 M lithium aluminum hydride (170.5 mL) was added dropwise at a rate to maintain reflux but avoid overflow. After addition was complete the reaction was heated to 80°C and refluxed for 48 hours. The reaction was quenched dropwise with deionized water, and combined with a solution of Rochelle’s Salt (150 mL) and stirred under nitrogen overnight. The solution was then filtered through celite, extracted with a 3:1 solution of methylene chloride:tetrahydrofuran (4 x 100 mL), dried over anhydrous sodium sulfate, and volatiles were removed under reduced pressure affording 11.3 g of crude free base. The free base was combined with 1.0 M HCl in diethyl ether (61 mL) forming a precipitate which was filtered and recrystallized overnight in the freezer with methanol/ethyl acetate affording 8.47g (67%) of pure white crystals. mp: 205.2-205.5°C; Anal. Calcd for C\(_{13}\)H\(_{15}\)N-HCl: C, 70.42; H, 7.27; N, 6.32. Found: C, 70.01; H, 7.39; N, 6.24. \(^1\)H NMR (CD\(_3\)OD) \(\delta\) (ppm): 7.86 (m; 3H, ArH), 7.73 (s; 1H, ArH), 7.50 (m; 2H, ArH), 7.39 (d; J = 1.7 Hz, 1H, ArH), 3.64 (X of ABX; m, 1H, CH\(_2\)CHCH\(_3\)NH\(_2\)), 3.13, 2.99 (AB of ABX; J\(_{AB}\) = 13.6 Hz, J\(_{AX}\) = 6.5 Hz, J\(_{BX}\) = 8.1 Hz, 2H, CH\(_2\)CHCH\(_3\)NH\(_2\)), 1.30 (d; J = 6.6 Hz, 3H, CH\(_2\)CHCH\(_3\)NH\(_2\)).

In vitro release assays.
In vitro $[^3\text{H}]\text{DA}$, $[^3\text{H}]\text{NE}$, and $[^3\text{H}]5\text{-HT}$ release assays were performed according to published methods (Rothman et al., 2002b). Briefly, a crude synaptosomal tissue preparation from rat caudate (DA release assay), or from whole brain minus caudate (NE and 5-HT release assays), was obtained by homogenization of freshly excised tissue in ice-cold 10% sucrose using 12 strokes of a Potter-Elvehjem homogenizer, followed by centrifugation at 1000g for 10 min. Supernatants were retained on ice and used immediately in release assays. All assays were performed in Krebs-phosphate buffer (pH 7.4) which contained 154.4 mM NaCl, 2.9 mM KCl, 1.1 mM CaCl$_2$, 0.83 mM MgCl$_2$, 5 mM glucose, 1 mg/ml ascorbic acid, and 50 µM pargyline. Reserpine (1 µM) was added to the sucrose solution and assay buffer. $[^3\text{H}]\text{NE}$ release assays were performed in the presence of 5 nM RTI-229 to prevent reuptake of $[^3\text{H}]\text{NE}$ into DAergic nerves. $[^3\text{H}]5\text{-HT}$ release assays were performed in the presence of 100 nM nomifensine and 100 nM GBR12909 to prevent reuptake of $[^3\text{H}]5\text{-HT}$ into NEergic and DAergic nerves. For release assays, synaptosomal preparations were incubated to steady state with 5 nM $[^3\text{H}]\text{DA}$ (30 min), 7 nM $[^3\text{H}]\text{NE}$ (60 min), or 5 nM $[^3\text{H}]5\text{-HT}$ (60 min). Synaptosomes preloaded with neurotransmitter were added to test tubes containing test drugs and incubated for 5 min ([$^3\text{H}]\text{DA}$ and [$^3\text{H}]5\text{-HT}$) or 30 min ([$^3\text{H}]\text{NE}$). At the designated time, the assay was filtered using a Packard Filtermate Harvester. Non-displaceable tritium was measured by incubations in the presence of 100 µM tyramine for $[^3\text{H}]5\text{-HT}$ release and 10 µM tyramine for $[^3\text{H}]\text{DA}$ and $[^3\text{H}]\text{NE}$ release. Data of three experiments were pooled and fit to the two parameter logistic equation of the best-fit estimates of the EC$_{50}$ and E$_{\text{max}}$ using MLAB-PC.

*In vivo microdialysis experiments.*
Surgical implantation of indwelling jugular catheters and intracerebral guide cannulae was carried out as described (Baumann et al., 2000). Guide cannulae were aimed at the prefrontal cortex according to coordinates ML-2.5 mm and AP+3.0 mm relative to bregma, DV-0.8 mm relative to dura. Rats were allowed one week to recover from surgery. On the evening prior to testing, extension tubes were connected to catheters, and microdialysis probes (3 mm x 0.5 mm exchange surface, CMA/12, CMA/Microdialysis) were inserted into the frontal cortex via guide cannulae. Each rat was attached to a tether and placed into a 40 cubic cm Plexiglass arena equipped with photobeams that allowed movements to be quantified (TruScan, Coulborn Instruments). The next morning dialysate samples were collected at 20 min intervals. Samples were immediately assayed for DA and 5-HT by microbore HPLC with electrochemical detection (ECD) as described elsewhere (Baumann et al., 2000). Three baseline samples, which differed by less than 10%, were collected, averaged, and all subsequent DA and 5-HT measures were expressed as a percent of the mean baseline. In vitro probe recoveries of DA and 5-HT ranged from 20-24%. Drug solutions were prepared immediately before use, and doses are expressed as the salt. The motor activity of rats was monitored during the dialysis sampling. Ambulation (i.e., forward locomotion) and stereotypy (i.e., repetitive movements) were quantified in 20-min bins and analysed. The neurochemical and locomotor data were evaluated statistically using one-factor (drug treatment) repeated measures analysis of variance (rANOVA).

Neurotoxicity studies.

Rats received 3 sequential doses of PAL-287 (18 mg/kg, i.p.), (+)-methamphetamine (6.0 mg/kg, i.p.), or (±)-MDMA (7.5 mg/kg, i.p.), one dose every 2 h.
Vehicle-treated rats received saline injections (1 ml/kg, i.p.) following the same schedule. Two weeks after the injections rats were sacrificed, brains were removed and various brain regions were dissected on ice. Cortical monoamine concentrations were determined by HPLC with ECD as described (Baumann et al., 2001).

Assays of human 5-HT<sub>2</sub> family receptors.

The activity of PAL-287 at cloned human 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub> and 5-HT<sub>2C</sub> receptors was conducted as described (Setola et al., 2003).

Drug self-administration studies carried out at the University of Mississippi Medical Center

Subjects and Apparatus.

The subjects were four male rhesus monkeys (Macaca mulatta) weighing between 10 and 10.7 kg at the beginning of the study. All the monkeys had histories of cocaine self-administration. Most recently, under the conditions of the present experiment, monkey AP78 had been tested with atomoxetine, monkey M341 with pseudoephedrine, and monkey AP01 with amphetamine analogs. Monkey 12884 was tested with cocaine and sibutramine under a progressive-ratio schedule (unpublished). All monkeys were provided with sufficient food to maintain stable body weight (120-180 g/day, Teklad 25% Monkey Diet, Harlan/Teklad, Madison, WI) and had unlimited access to water. Fresh fruit and a vitamin supplement were provided daily and three times a week, respectively.

The monkeys were individually housed in the experimental cubicles (1.0 m<sup>3</sup>, PlasLabs, Lansing, MI). Each monkey was fitted with a stainless-steel harness
attached by a tether to the rear wall of the cubicle. The front door of the cubicle was made of transparent plastic and the remaining walls were opaque plastic. Two response levers (PRL-001, BRS/LVE, Beltsville, MD) were mounted on the inside of the door. Four jeweled stimulus lights, two red and two white, were mounted above each lever. A peristaltic infusion pump (Cole-Parmer Co., Chicago, IL) delivered drug injections. A Macintosh computer with custom interface and software controlled all events in an experimental session.

**Procedure.**

Details of the procedure have been previously published (Wee et al., 2004). Briefly, monkeys were implanted with i.v. silastic catheters and allowed to self-administer drugs by pressing the right lever (25 lever presses/injection; FR 25) for two hours/day, beginning at noon, seven days/week. At the start of a session, the white lights were illuminated above both levers and responding on the right lever resulted in the delivery of a drug injection for 10 seconds. During the injection, the white lights were extinguished and the red lights were illuminated. Pressing the left lever was counted but had no other programmed consequence.

In baseline sessions, cocaine (0.03 mg/kg/injection for AP01, AP78, M341; 0.01 mg/kg/injection for L638) or saline was made available. Test sessions were arranged among baseline sessions in a way that a test session came after two different baseline sessions, that is, C-S-T or S-C-T where C, S and T denote cocaine, saline and test sessions, respectively. A test session was identical to a baseline session, except that saline or one of various doses of cocaine (0.001 - 0.3 mg/kg/injection) or PAL 287 (0.001- 0.3 mg/kg/injection) were made available for self-administration. After a test session, monkeys were returned to baseline conditions until cocaine- and saline-maintained responding were again stable. Doses were tested twice, once the session
after a cocaine baseline session and once the day after a saline baseline session. In monkey AP01, results of the two test sessions with dose of 0.03 mg/kg/injection PAL 287 varied widely. Therefore, this dose was made available for nine consecutive sessions until responding was stable.

Data analysis.

Raw data were analyzed as injections/session for individual monkeys. Means of two sessions were calculated for each dose of each drug, and the ranges of the two determinations served as the measure of individual variability. When mean injections/session at a dose of a drug was above the mean for saline sessions, and the ranges did not overlap, the dose of the drug was considered to function as a positive reinforcer.

Procedures carried out at the Alcohol and Drug Abuse Research Center, McLean Hospital

Subjects.

Studies were conducted in a group of 3 male rhesus monkeys (Macaca mulatta) implanted with chronic intravenous catheters under aseptic conditions. Monkeys weighing 8-12 kg and maintained on a diet of multiple vitamins, fresh fruit and Lab Diet Jumbo Monkey biscuits (PMI Feeds, Inc., St. Louis, MO). In addition, monkeys could receive 1 gm banana flavored pellets (P. J. Noyes Co., Lancaster, NH) during daily sessions (see below). Water was continuously available. A 12 hr light-dark cycle was in effect (lights on from 7 a.m. to 7 p.m.). The health of the monkeys was periodically monitored by consulting veterinarians. Monkeys had visual, auditory and olfactory contact with other monkeys throughout the study. Operant procedures and foraging
toys provided opportunities for environmental manipulation and enrichment. Music or nature video tapes were also played to provide additional environmental enrichment.

**Apparatus**

Each monkey was housed individually in a well-ventilated stainless steel chamber (66 x 76 x 94 cm) equipped with a custom-designed operant panel (28 x 28 cm) mounted on the front wall. Three round translucent response keys (diameter 5.1 cm) were arranged 2.54 cm apart in a horizontal row 3.2 cm from the top of the operant panel. Each key could be transilluminated by red or green stimulus lights (Superbright LED’s). In addition, three circular translucent panels (1.9 cm in diameter) were located in a vertical column below the center response key and could be transilluminated by red or green stimulus lights. Each housing chamber was also equipped with a pellet dispenser (Gerbrands, Model G5210, Arlington, MA) and two syringe pumps (Model B5P-IE, Braintree Scientific, Braintree, MA; or Model 980210, Harvard Apparatus, South Natick, MA), one for each lumen of the double lumen catheter. One syringe pump (the self-administration pump) was used to deliver cocaine injections. The second syringe pump (the treatment pump) was used to deliver saline or test drug as described below for each procedure. The treatment pump delivered injections every 20 min from 10:30 a.m. each day until 9:30 a.m. the next morning for a total of 3 injections/hr and 69 injections/day. No treatment injections were delivered between 9:30 a.m. and 10:30 a.m., and during this period, monkeys received their morning ration of food, and their health status was evaluated by the technical staff. Operation of the operant panels and data collection were accomplished with microprocessors and software purchased from Med Associates Inc. (Georgia, VT).

**Second-Order Schedule of Cocaine and Food Availability**
The effects of chronic PAL-287 administration on cocaine- and food-maintained responding under a second-order schedule were examined using procedures identical to those used in our previous studies of the effects of \(d\)-amphetamine treatment (Negus and Mello, 2003). Alternating daily sessions of food and cocaine availability were associated with different colored stimulus lights projected on the center response key of the operant response panel. Specifically, red stimulus lights signaled food availability and green stimulus lights signaled the availability of cocaine injections (delivered in a volume of 0.1 ml in 1 sec). Under the terminal schedule, the completion of a variable ratio of 16 responses on the center response key resulted in the illumination for 1 sec of an appropriately colored stimulus light (red for food, green for drug) underneath the center key (VR16:S). In addition, completion of this VR response requirement a fixed ratio of two times (FR2) resulted in delivery of the available reinforcer and the initiation of a 10 sec time-out period, during which the stimulus light illuminating the center response key was turned off and responding had no scheduled consequences. This terminal second-order schedule is designated as FR2(VR16:S). The two side keys were not transilluminated during sessions of food and cocaine availability, and responding on these keys had no scheduled consequences. Four food sessions and four drug sessions were conducted during each experimental day. Food sessions began at 6 a.m., 11 a.m., 3 p.m. and 7 p.m., and drug sessions began at 7 a.m., 12 noon, 4 p.m. and 8 p.m. At all other times, responding had no scheduled consequences. Each food and drug session lasted one hour or until 25 food pellets or 20 injections had been delivered, whichever occurred first. Thus, monkeys could earn a maximum of 100 food pellets per day and 80 injections per day. Studies were conducted seven days a week.
Testing Procedures

The effects of treatment with chronic saline and PAL-287 (0.1-1.0 mg/kg/hr) on food- and cocaine-maintained responding were compared during availability of 0.01 mg/kg/injection cocaine. These doses of PAL-287 were based on preliminary dose-ranging studies in this procedure. A unit dose of 0.01 mg/kg/injection cocaine was used, because (1) it was the lowest dose to reliably maintain high rates of cocaine self-administration in all monkeys, (2) we have shown previously that 0.01 mg/kg/injection cocaine and food maintain similar response rates in this procedure, and (3) previous studies have shown that behavior maintained by this unit dose of cocaine is sensitive to the effects of pretreatment compounds (e.g. (Negus and Mello, 2002; Negus and Mello, 2003a)). PAL-287 doses were tested in an irregular order across monkeys. Treatment with saline and each dose of PAL-287 was evaluated for a period of seven days. At the conclusion of each seven-day treatment period, the maintenance dose of cocaine (0.032 mg/kg/injection) and saline control treatment were reinstated for a period of at least four days and until the number of reinforcers per day maintained by cocaine and food returned to baseline levels. A supra-threshold dose of 0.032 mg/kg/inj cocaine is used as a maintenance dose during training and maintenance to assure that a high efficacy drug reinforcer (and not merely a threshold reinforcer) is used during training and maintenance phases of the experiment. This interval between successive treatments was designed to reduce the possibility of carry-over effects from one treatment condition to the next.

Data Analysis

The primary dependent variables were the number of cocaine injections (maximum=80 per day) and food pellets (maximum=100 per day) delivered per day. Data from the last three days of each 7-day treatment were used for data analysis.
Effects of PAL-287 on cocaine- and food-maintained responding were expressed as the percent of control levels of cocaine- and food-maintained responding observed during saline treatment. Data were analyzed by two-way ANOVA, with type of reinforcer (cocaine or food) and dose of PAL-287 (0.1-1.0 mg/kg/hr) as the two factors. A significant ANOVA was followed by the Newman-Keuls post hoc test. ED$_{50}$ values for PAL-287 were also determined for each monkey, with the ED$_{50}$ defined as the dose of PAL-287 that reduced cocaine- or food-maintained responding to 50% of control. A mean±SEM ED50 value was then determined, and ED$_{50}$ values for PAL-287 to reduce cocaine- and food-maintained responding were compared by T-test. For all statistical tests, the criterion for significance was set a priori at p<0.05.
Over 350 compounds were screened for activity as substrate-type releasing agents at DA transporters (DATs), 5-HT transporters (SERTs) and norepinephrine transporters (NET). These efforts identified PAL-287 (1-naphthyl-2-aminopropane) as a compound different from amphetamine that has potent actions in vitro as a substrate for DATs, SERTs and NETs (Fig. 1). The EC$_{50}$ values of PAL-287 at DAT (12.6±0.4 nM) and NET (11.1±0.9 nM) are similar to that reported for (+)-methamphetamine, 24.5±2.1 nM and 12.3 nM, respectively. However, the EC$_{50}$ value of PAL-287 at SERT (3.4±0.2 nM) is much lower than that of (+)-methamphetamine (736±45 nM) and amphetamine (1765±94 nM), and is about 10-fold lower than reported for 5-HT (44.4±5.3 nM) (Rothman et al., 2001).

As depicted in Fig. 2, in vivo microdialysis experiments demonstrated that i.v. administration of PAL-287 increased extracellular DA and 5-HT in rat prefrontal cortex in a dose-dependent manner. PAL-287 elevated 5-HT to a greater extent than DA at both doses tested. Figure 3 shows that i.v. (+)-amphetamine increased extracellular DA with minimal effects on 5-HT. It is noteworthy that 3 mg/kg of PAL-287 and 1 mg/kg (+)-amphetamine increased extracellular DA to similar extent, about 8-fold above baseline. Despite the large elevations in extracellular DA produced by PAL-287, the 3 mg/kg dose of the drug increased ambulation to a much lower level when compared to 1 mg/kg (+)-amphetamine (compare Figs. 4 and 5). Thus, PAL-287 displayed very weak locomotor stimulant activity.

The ability of PAL-287 to induce long-term depletion of brain 5-HT (i.e., neurotoxicity) was determined by administration of 3 sequential doses (18 mg/kg ip) every 2 hr. As positive controls, other rats received (+)-methamphetamine (6.0 mg/kg,
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ip) or (±)-MDMA (7.5 mg/kg, ip) administered according to the same schedule. Rats were sacrificed 2 weeks later and cortical 5-HT levels determined as described in Methods. The results (Fig. 6) indicated that whereas both (+)-methamphetamine and MDMA decreased cortical 5-HT, PAL-287 did not. None of the treatments depleted cortical DA (data not shown).

Using published established methods (Wee et al., 2004) four rhesus monkeys were trained to self-administer cocaine. As reported in Fig. 7, the cocaine dose-response curves in the monkeys followed a typical “inverted U” shape. PAL-287 (0.001-0.3 mg/kg/inj) displayed little or no reinforcing effect, indicative of a compound with low abuse potential. One of four monkeys self-administered PAL-287 when it was substituted for cocaine, whereas the other three did not.

Rhesus monkeys were trained to respond for food and cocaine injections as previously detailed (Negus and Mello, 2003b). Fig. 8 shows the effects of chronic, 7-day treatment with PAL-287 on cocaine- and food-maintained responding in three rhesus monkeys. During saline treatment, the mean number±SEM of cocaine injections per day was 79.4±0.6 (out of a maximum of 80), and the mean number of food pellets per day was 96.1±3.9 (out of a maximum of 100). PAL-287 produced a dose-dependent decrease in both cocaine- and food-maintained responding, and statistical results are reported in the figure legend. A dose of 1.0 mg/kg/hr PAL-287 significantly reduced both cocaine- and food-maintained responding; however, the suppression of cocaine self-administration was greater than the reduction in food-maintained responding. The ED_{50} values for PAL-287 to reduce cocaine- and food-maintained responding were 0.49±0.04 mg/kg/hr and 0.85±0.17 mg/kg/hr, respectively, and these values were not significantly different (p=0.19).
We also evaluated the ability of PAL-287 to activate cloned human 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors (see (Setola et al., 2003) for details). The results (Table 1) indicated that PAL-287 is a full agonist at the 5-HT_{2B} receptor (EC_{50} = 40 nM) and the 5-HT_{2A} receptor (EC_{50} = 466 nM). It is a potent partial 5-HT_{2C} agonist (EC_{50} = 2.3 nM, E_{MAX} = 20%). The partial agonist action of PAL-287 at the 5-HT_{2C} receptor predicts possible anorectic actions of PAL-287 (Vickers et al., 1999) and may also reduce the rewarding effects produced by the DA releasing actions of PAL-287 (see {Czoty, 2002 #11958} for a review). The relatively weak potency at 5-HT_{2A} and 5-HT_{2B} receptors, as compared to its activity at SERT, suggest that PAL-287 will have minimal functional actions at 5-HT_{2A} and 5-HT_{2B} receptors in vivo.
Discussion

The main purpose of the present study was to identify a novel monoamine releaser that could be developed as an agonist replacement medication for treating stimulant dependence. We chose to focus our attention on a non-amphetamine molecule with dual DA/5-HT releasing properties for several reasons. First, a wealth of information supports the existence of a dual deficit in synaptic DA and 5-HT during withdrawal from chronic stimulant abuse. A medicine that releases DA and 5-HT might alleviate stimulant withdrawal symptoms by restoring neurochemical deficits in abstinent addicts. Second, DA releasers (e.g. phentermine) and 5-HT releasers (e.g. fenfluramine) are both known to suppress ongoing stimulant self-administration in animal models. By designing a single molecular entity with mixed DA/5-HT releasing activity, two separate mechanisms could be recruited to suppress illicit stimulant use. Finally, we predicted that a dual DA/5-HT releaser would have reduced abuse liability when compared to a selective DA releaser, since 5-HT release can dampen the stimulant effects mediated by DA. Our results with PAL-287 confirm the hypothesis that a non-amphetamine substrate at DA transporters (DATs) and 5-HT transporters (SERTs) will release DA and 5-HT from neurons in vivo, and thereby suppress ongoing cocaine self-administration. PAL-287 displays a number of desirable qualities for a candidate medication, including minimal locomotor activation, lack of long-term neurotoxicity, and low abuse potential.

Preclinical and human data suggest that withdrawal from chronic cocaine is associated with impairments in 5-HT neuronal function, in addition to the well accepted deficits in DA function (Dackis and Gold, 1985). Perhaps the most compelling evidence for 5-HT deficits in cocaine addiction is the occurrence of a psychiatric syndrome resembling major depression following abstinence from binge cocaine use (Dackis and
Gold, 1985; Gawin and Kleber, 1986), coupled with the increased prevalence of suicidal ideation and suicide attempts among cocaine addicts (Garlow et al., 2003). The well established role of 5-HT dysfunction in mediating depression and suicide (for review see (Mann, 2003)) suggests that decreased synaptic 5-HT might play a role in cocaine withdrawal states. In support of this notion, rats withdrawn from chronic cocaine treatment display abnormalities in 5-HT-mediated neuroendocrine secretion that are similar to those observed in humans diagnosed with major depression (Baumann and Rothman, 1998).

We have previously proposed a dual deficit model of stimulant withdrawal where drug-induced decreases in synaptic DA and 5-HT contribute to withdrawal dysphoria, drug craving, and relapse (for review see (Baumann et al., 2000)). Initial observations in human patients receiving the DA releaser phentermine and the 5-HT releaser fenfluramine as medications, showed that concurrent DA and 5-HT release can ameliorate symptoms of cocaine withdrawal and reduce illicit cocaine use (Rothman et al., 1994; Kampman et al., 2000). These preliminary clinical findings strengthened the rationale for development of DAT and SERT substrates as potential treatments for stimulant addiction.

The present data with PAL-287 support the use of monoamine releasers as agonist-substitution medications for the treatment of stimulant addictions. A dose of 1.0 mg/kg/hr PAL-287 virtually eliminated cocaine self-administration in rhesus monkeys by the end of the seven-day treatment, although this effect was not selective. The significant decreases in food-maintained responding suggest that decreases in cocaine self-administration may have resulted, at least in part, from non-selective effects of PAL-287 that limited the ability of the monkeys to emit operant responses. It should be noted however that cocaine self-administration was virtually eliminated by 1.0 mg/kg/hr PAL-
287, whereas food-maintained was decreased by 65%, and cocaine self-administration
was decreased more than food-maintained responding in all three monkeys. These
results demonstrate that chronic, seven-day treatment with PAL-287 produced a dose-
dependent and moderately selective decrease in cocaine self-administration in rhesus
monkeys responding under a second-order schedule. Future studies should examine
the effect of PAL-287 on other unit doses of cocaine.

Our findings with PAL-287 in monkeys are reminiscent of the suppression of
cocaine self-administration produced by (+)-amphetamine reported previously, although
amphetamine displays greater selectivity than PAL-287 in reducing cocaine self-
administration as opposed to food-maintained responding (Negus and Mello, 2003b).
Grabowski et al. (Grabowski et al., 2001; Grabowski et al., 2004) showed that a slow-
release formulation of (+)-amphetamine is effective in maintaining cocaine addicts in
treatment and reducing illicit cocaine use. We predict that agents like PAL-287, which
have mixed DA/5-HT releasing activity, will possess the therapeutic effects of
amphetamine-type monoamine releasers, while minimizing the adverse effects
associated the phenethylamine structure. The role of NE in the actions of PAL-287 is an
important issue awaiting additional study (Rothman et al., 2001).

It is interesting to note that PAL-287 and PAL-313 (para-methylamphetamine),
the reinforcing effects of which were reported in a recent paper (Wee et al., 2005), are
both equipotent as releasers across the monoamines. Yet PAL-287 was not a reinforcer
under a simple FR10 schedule, whereas PAL-313 was a reinforcer under a progressive
ratio schedule where the response requirement was increased to at least 200 lever
presses per injection. Additional research will be required to determine why these two
agents differ in their reinforcing efficacy.
One limitation of using amphetamine-type drugs as medications is their high abuse potential, which is related to stimulation of DA release in mesolimbic reward pathways. Several lines of evidence support the hypothesis that elevations in synaptic 5-HT can counteract the stimulant and reinforcing effects mediated by elevations in synaptic DA (Daw et al., 2002; Burmeister et al., 2004). For example, 5-HT precursor loading with dietary tryptophan attenuates amphetamine self-administration in rats (Smith et al., 1986). Other studies show that pretreatment with 5-HT reuptake inhibitors reduces intravenous cocaine self-administration in rats (Carroll et al., 1990) and squirrel monkeys (Howell and Byrd, 1995). Consistent with these data, cocaine analogs that have enhanced 5-HT transporter affinity (Roberts et al., 1999) support less self-administration behavior than analogs with lower 5-HT transporter affinity. The comparative microdialysis results reported here for PAL-287 and (+)-amphetamine provide direct support for the hypothesis that 5-HT release dampens stimulant effects mediated by DA release. PAL-287 (3 mg/kg, i.v.) and (+)-amphetamine (1 mg/kg, i.v.) increased extracellular DA to a similar extent, but the locomotor stimulant effects of PAL-287 were much less than those of (+)-amphetamine. We believe that reduced locomotor activation afforded by PAL-287 is related to the marked increase in extracellular 5-HT produced by this drug, an effect which is not shared by the selective DA releaser (+)-amphetamine.

A number of additional side effects could limit the use of dual DA/5-HT releasing agents. For example, some 5-HT releasing agents cause adverse effects that include neurotoxicity, cardiac valvulopathy and primary pulmonary hypertension (PPH) (for review see: Rothman and Baumann, 2002). Our recent investigations support the possibility of developing dual DA/5-HT releasers that lack adverse effects. In particular, it was suggested that a lead drug molecule should be chemically distinct from the
phenethylamine structure shared by amphetamine-like agents because substituted phenethylamines are associated with neurotoxicity in animals. Furthermore, the candidate medication should lack significant interaction at the 5-HT$_{2B}$ receptor, a site which is implicated in pathogenesis of fenfluramine-associated cardiac valvulopathy (Fitzgerald et al., 2000; Rothman et al., 2000; Setola et al., 2003).

The term ‘5-HT neurotoxicity’, when used in the present context, refers to the fact that high-dose administration of selective 5-HT releasers (e.g. fenfluramine) often causes persistent depletion of brain tissue 5-HT and 5-HT transporters. A key observation is that not all SERT substrates deplete 5-HT, since repeated administration of the SERT substrate m-chlorophenylpiperazine (mCPP) fails to deplete brain 5-HT, despite producing elevations of extracellular 5-HT comparable to fenfluramine (Baumann et al., 2001). Like mCPP, PAL-287 produces elevations in extracellular 5-HT but does not produce long-term 5-HT depletion, even after exposure to very high doses (i.e., 18 mg/kg, i.p. x 3). These data indicate that SERT substrate activity is necessary, but not sufficient to produce long-term depletion of brain 5-HT. Increasing evidence suggests that SERT sites are involved in the mechanism by which fenfluramine increases the risk of developing PPH (for review see (Rothman and Baumann, 2002) and references therein). Medications that increase the risk for PPH share activity as SERT substrates. On the other hand, not all SERT substrates are associated with PPH. The antidepressant trazodone is not associated with PPH, yet its major metabolite, mCPP is a potent SERT substrate as noted above (Baumann et al., 2001).

PAL-287 displayed full agonist actions at the 5-HT$_{2B}$ and 5-HT$_{2A}$ receptors, but the potency of PAL-287 at these receptors was at least ten-fold lower than its potency as a SERT substrate (3.4 nM). It is important to recognize that drug potency estimates determined from in vitro expression systems may overestimate the potency of drugs at
5-HT receptor subtypes, due to a variety of confounding factors (i.e., protein expression level) that are not characteristic of native tissue (Bockaert et al., 1997). Although our data suggest that PAL-287 will not significantly activate 5-HT\textsubscript{2B} and 5-HT\textsubscript{2A} receptors in vivo, further studies will be needed to address this question more directly.

The use of stimulant-like medications to treat stimulant addictions is an approach described as “agonist substitution” therapy. This strategy involves administering medications which are less potent and less addictive than cocaine or methamphetamine, but that nevertheless decrease stimulant abuse because of shared neurochemical properties with the illicit drugs (Gorelick, 1998). Viewed from this perspective, agonist substitution therapy can be described as “neurochemical normalization” therapy - the treatment medication “normalizes” dysregulated neurochemistry by substituting for the abused drug. This approach has been explored for the treatment of cocaine dependence (Alim et al., 1995; Grabowski et al., 1997; Kampman et al., 2000; Walsh et al., 2000; Grabowski et al., 2001). Our results with PAL-287 extend the concept of agonist substitution by employing a dual DA/5-HT releaser that exhibits the desired therapeutic effects of a replacement medication without the adverse side-effects associated with prototypical psychomotor stimulants.

In summary, the findings reported here demonstrate that a non-amphetamine monoamine releasing agent with low abuse potential and minimal neurotoxicity can suppress cocaine self-administration in sub-human primates. As such, PAL-287 represents the prototype for a new generation of drugs that enhance biogenic amine release by acting as substrates at multiple biogenic amine transporters (DATs, SERTs, and NETs). It seems possible that drugs with a similar mode of action will provide neurochemical normalization therapy for the treatment of cocaine addiction, and also be
potentially useful for treating depression, obsessive compulsive disorder, attention deficit disorder and obesity.
Acknowledgements

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the need for treatment paradigms for drug-abusing patients with multiple


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norepinephrine more potently than they release dopamine and serotonin.


Footnotes:

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Figure 1: Dose-response curves showing PAL-287-induced release of $[^3]$Hneurotransmitter via the DAT, NET, and SERT. The curves shown are one representative experiment. The data of 3 experiments were pooled and fit to the two parameter logistic equation for the best fit estimate of the EC$_{50}$ (nM ± SD), reported in the text. The structure of PAL-287 is shown in the inset.

Figure 2: Dose-response effects of PAL-287 on extracellular DA and 5-HT in rat prefrontal cortex as determined by in vivo microdialysis. Rats received i.v. injection of 1 mg/kg PAL-287 at time zero, followed by 3 mg/kg 60 min later. Data are mean ± SEM for 7 rats/ group, expressed as % baseline. Baseline levels of DA and 5-HT were 0.43 ± 0.07 and 0.27 ± 0.06 pg/5 µl. * * P<0.05 compared to pre-injection control, Duncan’s post hoc test.

Figure 3: Dose-response effects of (+)-amphetamine on extracellular DA and 5-HT in rat prefrontal cortex as determined by in vivo microdialysis. Rats received i.v. injection of 0.3 mg/kg (+)-amphetamine at time zero, followed by 1 mg/kg 60 min later. Data are mean ± SEM for 7 rats/ group, expressed as % baseline. Baseline levels of DA and 5-HT were 0.38 ± 0.07 and 0.24 ± 0.06 pg/5 µl. * * P<0.05 compared to pre-injection control, Duncan’s post hoc test.

Figure 4: Dose-response effects of PAL-287 on ambulation and stereotypy in rats undergoing microdialysis sampling. Rats received i.v. injections of 1 mg/kg PAL-287 at time zero, followed by 3 mg/kg 60 min later. Data are mean ± SEM for 7 rats/ group,
expressed as distance traveled in cm (ambulation) and number of repetitive movements (stereotypy). * * P<0.05 compared to pre-injection control, Duncan’s post hoc test.

Figure 5: Dose-response effects of (+)-amphetamine on ambulation and stereotypy in rats undergoing microdialysis sampling. Rats received i.v. injections of 0.3 mg/kg (+)-amphetamine at time zero, followed by 1 mg/kg 60 min later. Data are mean ± SEM for 7 rats/ group, expressed as distance traveled in cm (ambulation) and number of repetitive movements (stereotypy). * P<0.05 compared to pre-injection control, Duncan’s post hoc test.

Figure 6: Effects of high-dose administration of PAL-287 on long-term 5-HT depletion in rat brain. Rats received 3 sequential i.p. injections of PAL-287 (18 mg/kg), (+)-methamphetamine (METH; 6.0 mg/kg), MDMA (7.5 mg/kg) or saline, one injection every two h. Rats were sacrificed 2 weeks after injections and brain tissue levels of 5-HT and 5-HIAA were determined by HPLC with ECD. Data are mean ± SEM for N=6 rat/ group, expressed as % baseline. * P<0.05 compared to saline-injected control, Duncan’s post hoc test.

Figure 7: Self-administration of cocaine and PAL 287 by rhesus monkeys. Drugs were available under a FR 25 schedule of reinforcement for two hours/day. Each point is the mean of two sessions of access to each dose of the drugs. Data are mean ± SEM for N=4 monkeys. Symbols without bars have variability smaller than the points. * P<0.05 compared to saline-injected control, Duncan’s post hoc test.
Figure 8: Effects of chronic, 7-day treatment with PAL-287 on cocaine-and food-maintained responding. Abscissa: Dose PAL-287 in mg/kg/hr (log scale). Ordinate: Percent control levels of cocaine- and food-maintained responding. Control values were defined as levels of cocaine- or food-maintained responding observed during 7 days of saline treatment (79.4±0.6 cocaine injections per day out of a maximum of 80; 96.3±3.9 food pellets per day out of a maximum of 100). Each point shows mean data±SEM for three monkeys collected during the last three days of each seven-day treatment. Two-way ANOVA indicated a significant effect of PAL-287 dose [F(2,4)=167, p=0.0001], but not a significant effect of reinforcer type [F(2,2)=7.26, p=0.114] or a significant interaction [F(2,4)=3.86, p=0.116]. Post hoc analysis was conducted with the Newman-Keuls test. * Indicates significant effect of PAL-287 dose in comparison to control for a given reinforcer, p<0.05. ¥ Indicates that food-maintained responding was significantly greater than cocaine-maintained responding at that dose of PAL-287, p<0.05.
### Table 1

Summary of Studies Conducted with Cloned Human Serotonin Receptors

<table>
<thead>
<tr>
<th></th>
<th>h5-HT$_{2A}$</th>
<th>h5-HT$_{2B}$</th>
<th>h5-HT$_{2C}$</th>
<th>h5-HT$_{2C}$ (VGI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT EC50 (nM); (pEC50±SD)</td>
<td>13 (1.13 ± 0.08)</td>
<td>2.5 (0.39 ± 0.19)</td>
<td>1.2 (0.07 ± 0.18)</td>
<td>2.0 (0.29 ± 0.1)</td>
</tr>
<tr>
<td>E$_{max}$</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PAL-287 EC50 (nM); (pEC50±SD)</td>
<td>466 (2.7 ± 0.08)</td>
<td>40 (1.6 ± 0.2)</td>
<td>2.3 (0.36 ± 0.44)</td>
<td>102 (2.01 ± 0.20)</td>
</tr>
<tr>
<td>E$_{max}$</td>
<td>74%</td>
<td>91%</td>
<td>20%</td>
<td>35%</td>
</tr>
</tbody>
</table>

*Studies measured drug effects on Phosphoinositide hydrolysis. *Studies measured drug effects on VGI. Each value is the mean±SD (n= 3).
Figure 1

[Graph showing the concentration-response relationship for 5-HT, DA, and NE with PAL-287.]
Figure 3

![Graph showing dialysate amine levels over time with different treatments (DA and 5-HT) and time points (0.3 mg/kg and 1 mg/kg)].

- O - DA
- ■ - 5-HT

Dialysate Amine (% baseline)

Time (min)

0 40 0 80 120

0 400 800 1200

0.3 mg/kg

1 mg/kg

* * * * *
Figure 4

The graph shows the effect of 1 mg/kg and 3 mg/kg doses on ambulation and stereotypy. The x-axis represents time in minutes, and the y-axis represents the number of events for ambulation and stereotypy. The graph includes arrows indicating the administration of the doses and asterisks denoting statistically significant differences. The data suggest that higher doses lead to increased ambulation and decreased stereotypy.
Figure 5

The graph depicts the time course of ambulation and stereotypy following drug administration. The x-axis represents time in minutes, ranging from -40 to 120. The y-axis for ambulation is labeled in centimeters (cm) from 0 to 8000, and the y-axis for stereotypy is labeled in events from 0 to 2000.

- **Ambulation**: Represented by open circles. There is a significant increase in ambulation at 0.3 mg/kg and further at 1.0 mg/kg.
- **Stereotypy**: Represented by filled squares. There is a significant increase in stereotypy at 0.3 mg/kg and further at 1.0 mg/kg.

* indicates a significant difference from the baseline.
Figure 6

![Bar graph showing tissue indole levels in different conditions.]

- Saline
- PAL-287
- METH
- MDMA

Tissue Indole (% control)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cortex</th>
<th>5-HT</th>
<th>5-HIAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAL-287</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>METH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDMA</td>
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</tbody>
</table>

* Indicates statistically significant difference.
Figure 8

![Graph showing the effect of different doses of PAL-287 on percent control of cocaine (0.01 mg/kg/inj) and food (1 gm pellets). The graph indicates a decrease in percent control as the dose of PAL-287 increases.](image-url)